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14-3-3 Proteins and photoneuroendocrine transduction: role in controlling the daily rhythm in melatonin

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Abstract

This paper describes the role 14-3-3 proteins play in vertebrate photoneuroendocrine transduction. 14-3-3 proteins form a complex with arylalkylamine N-acetyltransferase (AANAT), the enzyme which turns melatonin production on during the day and off at night. Complex formation is triggered at night by cAMP-dependent phosphorylation of the enzyme, and results in activation and protection against proteolysis. This enhances melatonin production > 10-fold. Light exposure results in dephosphorylation of the enzyme and disassociation from 14-3-3, leading to destruction and a rapid drop in melatonin production and release and circulating levels.

Key words: cAMP-dependent protein kinase, N-acetyltransferase, pineal.

Abbreviations used: AANAT, arylalkylamine N-acetyltransferase; PKA, cAMP-dependent protein kinase.

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Introduction

Life scientists are intrigued by changes in biological systems and what regulates them. Accordingly, it is not surprising that the large, rapid and precisely regulated physiological changes in melatonin and arylalkylamine N-acetyltransferase (AANAT; serotonin N-acetyltransferase or NAT, EC 2.3.1.87; Figure 1) in the pineal gland have fascinated investigators. AANAT is the first enzyme in the serotonin ('5-hydroxytryptamine') → *N*-acetylserotonin → melatonin pathway and plays a unique regulatory role in vertebrates by controlling the conversion of night-time darkness into the hormonal signal of melatonin [1–3]. In some species AANAT activity increases > 100-fold at night. In the early 1990s we were intent on purifying this enzyme, as a step towards cloning and a better understanding of AANAT. Accordingly, there was some disappointment when two promising AANAT candidate proteins in fractions highly enriched with AANAT activity were found to be members of an unfamiliar family, the 14-3-3 proteins; we wrote "... it is possible they could regulate NAT activity; this remains to be demonstrated ..." [4]. Eventually we cloned AANAT [5,6] and soon were brought face-to-face with 14-3-3 proteins again [7], which led to the discovery that an AANAT–14-3-3 complex plays a key role in regulating melatonin production [7,8]. Highlights of these interesting developments are covered in this article, which also provides the reader with an overview of the role AANAT plays in vertebrate biology, so that the importance of the AANAT–14-3-3 complex can be considered in a physiological context.

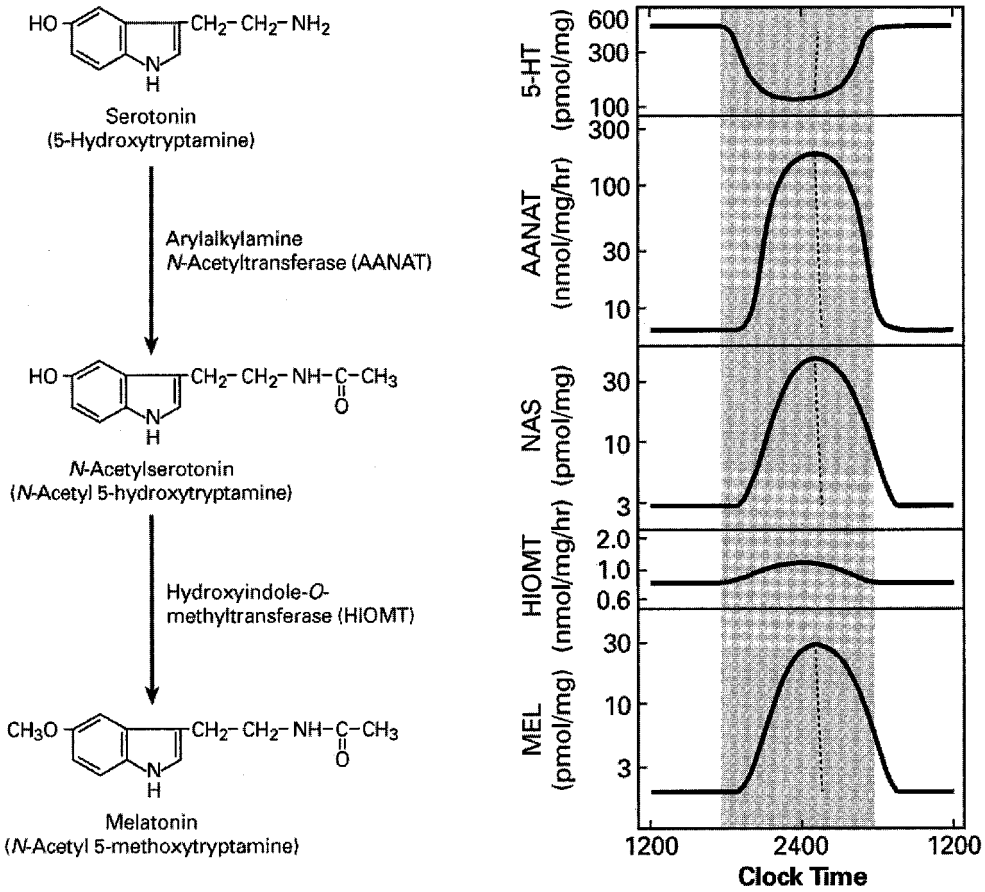
Photoneuroendocrine transduction by the pineal gland

The term photoneuroendocrine transduction describes the process through which signals about

Figure 1

Daily rhythm in pineal indoles

The serotonin → *N*-acetylserotonin → melatonin pathway is shown on the left; changes in levels of the compounds and enzymes is shown on the right. The dark panel represents darkness; the dotted line represents changes due to exposure to light at night. 5-HT, 5-hydroxytryptamine; NAS, *N*-acetylserotonin; HIOMT, hydroxyindole O-methyltransferase; MEL, melatonin.



environmental lighting, day and night, are converted into a hormonal signal. Animals tell time this way, much as we use a watch. Typically, photoneuroendocrine transduction systems have three elements. One is a means of detecting light; the second is an autonomous ≈ 24 h (circadian) clock, which is synchronized to the environmental day/night cycle by light; and the third is the capacity to generate an output signal that is an analogue of duration of the dark period, in this case melatonin. The endogenous nature of the clock allows animals to anticipate changes in the environment and to optimize the synchronization of physiological functions with seasonal and daily changes in the environment. For example, this allows animals in dark burrows to accurately predict dawn, and enables animals to reproduce on a seasonal schedule – anticipating spring – which increases the opportunity for newborns to survive.

Melatonin as the hormone of the night

The two most obvious features of melatonin dynamics are the 24 h rhythm (values are low during the day and high at night) and the dramatic suppressive effects of light exposure at night (Figure 1). The close association of melatonin with night has earned melatonin the title of the hormone of the night.

Circulating melatonin levels reflect the balance between production in the pineal gland and destruction by the liver [1,9]. Dynamic changes in circulating melatonin levels are caused by changes in synthesis; the potential for hepatic destruction is always high. This high level of destruction is important for two reasons: it maintains circulating melatonin at low levels during the day and allows the termination of melatonin synthesis to be immediately translated into a decrease in circulating melatonin, thereby improving the 'square wave' quality of the melatonin signal.

Melatonin does not have a single target system [1], in contrast with some hormones. The temporal information it provides is used differently by different species and tissues to optimize circadian and seasonal physiology.

Regulation of melatonin production: the essential role of AANAT

High levels of AANAT activity at night cause serotonin levels to decrease and *N*-acetylserotonin levels to increase. The increase in *N*-acetylserotonin increases melatonin production by a mass-action effect on the last enzyme in melatonin synthesis, hydroxyindole O-methyltransferase, i.e. more substrate leads to more product (Figure 1). The levels of active hydroxyindole O-methlytransferase protein remain relatively constant over the course of a day, although they change gradually over a period of days in response to changes in lighting [9].

cAMP plays a central role in regulating melatonin production through effects on AANAT activity. It acts in several ways; the most highly conserved and common mechanism is to prevent proteasomal proteolysis [10–13]. Less conserved mechanisms include effects on gene expression; for example, cAMP turns on AANAT gene expression in rodents causing a > 100-fold increase in AANAT mRNA [9,14], but plays little or no importance in regulating AANAT mRNA in birds [15], or in ungulates and non-human primates, in which AANAT mRNA remains relatively constant [5,6]. Another less well understood action of cAMP involves stimulating acetylation of substrate without altering the amount of existing AANAT protein [9,16].

There is remarkable diversity among vertebrate classes in the organization of melatonin rhythm-generating systems [17]. In mammals melatonin production is turned on and off by a neural system, that includes a circadian oscillator located in the suprachiasmatic nucleus of the hypothalamus [18]. Light acts on the suprachiasmatic nucleus via the retina and a retinal-hypothalamic projection [2,9]. This projection resets the clock and, through an independent mechanism, gates transmission of signals from the suprachiasmatic nucleus to the pineal gland [1,2,19]. The suprachiasmatic nucleus and pineal gland are linked by a neural pathway which passes through central and peripheral neural structures; at night noradrenaline is released into the pineal perivascular space, where it diffuses to the surface of the pinealocyte and activates adrenergic receptors to increase cAMP and other second messengers [9].

In contrast to the multi-component mammalian melatonin rhythm-generating system, that in fish and birds is located entirely in the pinealocyte [17], which contains photoreceptors and a circadian clock, in addition to the capacity to make melatonin. Accordingly, cultured fish and bird pineal glands maintain circadian rhythms of melatonin production and also respond to light.

The diverse nature of the anatomical organization of melatonin rhythm-generating systems is

in sharp contrast to the highly conserved nature of the cAMP-AANAT mechanism involved in regulating melatonin production, which appears to have been operating in primitive ancestral vertebrates. This is a reflection of the fundamental importance that this photoneuroendocrine transduction mechanism plays in vertebrate biology.

AANAT

Superfamily relationships

AANAT is a member of the very large GCN5 (motif A/B) superfamily of acetyltransferases, containing over 100 members [19,20]. GCN5 refers to an important member of this superfamily, a histone acetyltransferase. The term motif A/B refers to two conserved sequences that identify family members. The acetyl donor used by all members is acetyl CoA, which binds to a conserved pocket containing these motifs; in contrast to this conservation, there is marked variation in those regions of acetyltransferases that bind acetyl acceptors (i.e. serotonin, histones), consistent with their diversity [20]. Other members of this superfamily are not known to bind directly to 14-3-3 proteins.

The AANAT gene

The AANAT gene occurs in vertebrates and yeast; hypothetical AANATs also have been identified in bacteria [5,6,21]. Published genomes of plants, nematode worm or fruit fly do not contain the gene, which has led to the proposal that AANAT may have been introduced into vertebrates by the highly controversial and rare mechanism of lateral/horizontal transfer [21]. A single gene encodes AANAT in all vertebrates except fish, where genome duplication has resulted in two AANAT genes, AANAT-1 and AANAT-2 [22]. AANAT is expressed at high levels only in the pineal gland and to a variable degree in the retina [6], the second tissue known to synthesize melatonin. Expression in other tissues is negligible. In fish, AANAT-1 is preferentially expressed in the retina and AANAT-2 in the pineal gland.

The AANAT protein

Vertebrate AANAT is soluble cytosolic $\approx$ 24 kDa protein ($\approx$ 207 residues; Figure 2); yeast AANAT is $\approx$ 23 kDa proteins ($\approx$ 191 residues) [5,6,21]; high homology exists between 165 residues of the central catalytic cores of yeast and vertebrate AANAT. There are, however, notable differences in the flanking regulatory domains: yeast AANAT does not contain the consensus cAMP-dependent protein kinase (PKA) phosphorylation sites (PKA sites) found in the C- and N-terminal flanking regions of vertebrate AANAT [5,6] (Figure 2). The N-terminal PKA site (underlined below) is embedded within a sequence that becomes a consensus 14-3-3-binding motif [23–27] upon phosphorylation (RRHTLPAN $\rightarrow$ RRHpT LPAN; where pT is phosphothreonine).

Mechanism of acetyl transfer: catalytic and structural features

The kinetics of acetyl transfer by AANAT follow an ordered BiBi ternary complex scheme in which acetyl CoA binding occurs first [28]. Binding of the arylalkylamine (e.g. serotonin) and deprotonation leads to nucleophilic attack on the neighbouring thioester bond of acetyl CoA. This results in the formation of an unstable arylalkylamine–acetyl CoA intermediate [28,29], which yields acetylated arylalkylamine (e.g. *N*-acetylserotonin) and CoA. The hydrophilic/hydrophobic change of the arylalkylamine caused by acetylation favours ejection of the product from the active-site domain; release of CoA may be facilitated by reprotonation of the thiolate.

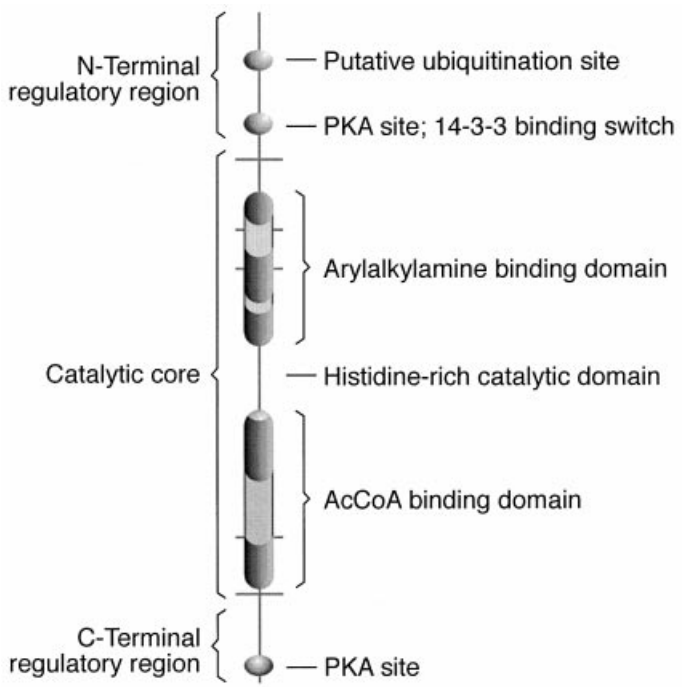
Catalysis is accompanied by a conformational change [28,30,31], which is of special interest because this is influenced by 14-3-3, as discussed below. The change involves a loop structure in the protein loop 1, that undergoes reorganization [30,31]. In the absence of substrates and cofactor, the loop can extend into the acetyl CoA-binding pocket, thereby blocking acetyl CoA binding. This conformational change was predicted first from kinetic analysis [28] and then became clear from structural analysis of the AANAT crystal obtained with and without a bisubstrate inhibitor (CoA–*S*-acetyltryptamine) that mimics the transition state intermediate [29–31]. In the absence of substrates, loop 1 extends into the acetyl CoA-binding domain; during catalysis, it undergoes extensive reorganization so as to allow acetyl CoA to bind. In this configuration, loop 1 also completes the arylalkylamine-binding domain [31], by adding a hydrophobic ring that assists in positioning the arylalkylamine substrate. The flexibility of loop 1 appears to enhance the rate of catalysis, perhaps by facilitating movement of one or more of the reactants during the catalytic cycle.

It is of interest that loop 1 is shorter in yeast AANAT compared with that in vertebrate AANAT; this may contribute to the lower V_{max} values of the yeast enzyme [21].

Figure 2

Schematic representation of vertebrate AANAT

The central catalytic core is flanked by regulatory regions, each of which has a PKA site. The N-terminal PKA is located within a sequence that is converted to a 14-3-3-binding motif upon phosphorylation (RRHTLPAN → RRHpTLPAN).



The AANAT–14-3-3 complex

History

As indicated in the Introduction, the association of 14-3-3 and AANAT was first recognized serendipitously in efforts aimed at the purification of AANAT [4]. A three-step chromatographic procedure (disulphide exchange, ion exchange and gel exclusion) generated fractions ($\approx$ 100 kDa) that were enriched in AANAT activity. Microsequencing of the two most prominent bands of AANAT candidate proteins revealed that they were primarily 14-3-3 ε and ζ. 14-3-3 was not pursued at the time because of an overriding interest in AANAT.

Cloning of AANAT [5,6] made it possible to develop antisera against the enzyme, which led to the discovery that AANAT activity and AANAT protein change in parallel in several experimental systems [6,10]. The rapid decrease in AANAT activity caused by light exposure at night (Figure 1) or following adrenergic receptor blockade is associated with a similar decrease in enzyme protein. Related studies [10] established that this was due to proteasomal proteolysis. Of special importance was the finding that the rapid decrease in enzyme protein was prevented by cAMP, which pointed to the potential importance of the two PKA sites in AANAT for the regulation of enzyme destruction.

We pursued hypothetical AANAT-binding proteins using a differential affinity chromatographic approach (AANAT versus phosphorylated AANAT), which resulted in identification of only two bands of proteins that exhibited strong phosphorylation-dependent binding [7]. Mass spectrographic analysis of tryptic fragments revealed that they contained 14-3-3 ε, ζ and γ, and perhaps β and θ. This indicated that AANAT–14-3-3 association was highly selective. The strength of the association was evident from both the results of differential affinity chromatography and the earlier multi-step chromatographic work [4].

Formation of a complex by expressed 14-3-3 and phosphorylated AANAT

Purified bacterially expressed 14-3-3 ζ protein and AANAT form a complex if AANAT is phosphorylated by PKA [7]. Complex formation is reversed and prevented by a 14 amino acid phosphopeptide that includes the N-terminal PKA/14-3-3-binding motif of AANAT; the unphosphorylated peptide does not have this effect.

This indicates that formation of the complex involves the PKA/14-3-3-binding motif. Phosphopeptides containing this motif bind to an amphiphathic groove of 14-3-3 [23–27,33], pointing to the probability that AANAT binds to this site via the PKA/14-3-3-binding motif. The composition of the complex is a single molecule of phosphorylated AANAT and a 14-3-3 dimer (1:1 dimer), according to chromatographic analysis [7].

Formation of the 14-3-3–AANAT complex in cells

The 14-3-3–AANAT complex forms in non-pineal cell lines that express the AANAT gene [7]. cAMP treatment enhances the abundance of the complex in these cell lines, consistent with the conclusions that formation is dependent upon PKA phosphorylation, that formation of the complex causes AANAT to accumulate and that pineal-specific factors are not required for complex formation to occur in intact cells. AANAT in the complex formed in cell lines or pineal cells is in a phosphorylated state. Together these findings support the conclusion that AANAT–14-3-3 binding is regulated by phosphorylation of the N-terminal PkA site and that binding occurs through the N-terminal PKA/14-3-3-binding motif.

Structure of the 14-3-3 ζ–AANAT complex

The three-dimensional structures of the molecules in the crystallized complex are generally similar to those of the uncomplexed proteins [8,33–35]. AANAT is bound to the interior surface of the dimer, in the central channel (Figure 3). This is associated with slight movement of the 14-3-3 monomers relative to each other, so that there is increased opening of the central groove. As predicted, there are multiple contacts between the PKA/14-3-3 motif of AANAT and the amphipathic groove of 14-3-3. These contacts are nearly identical to those involved in phosphopeptide binding. Multiple contacts between the phosphate group of Thr-31 and 14-3-3 explains why phosphorylation is the critical determining factor controlling formation of a stable complex. In addition to these contacts, others occur and contribute to the stability of the complex. However, these clearly are not sufficient to form a stable complex if AANAT is not phosphorylated.

The most important structural consequence of binding is not immediately apparent from examination of the static structures of the crystallized proteins. However, it becomes evident when flexibility of loop 1 of AANAT is considered. As indicated above, this loop is mobile in the absence of substrates and appears to move during the catalytic cycle. Analysis of the structure of the AANAT–14-3-3 complex indicates that movement of loop 1 is likely to be restricted by contacts with the 14-3-3 partner so as to prevent the loop from assuming the configuration in which it occupies in the absence of acetyl CoA.

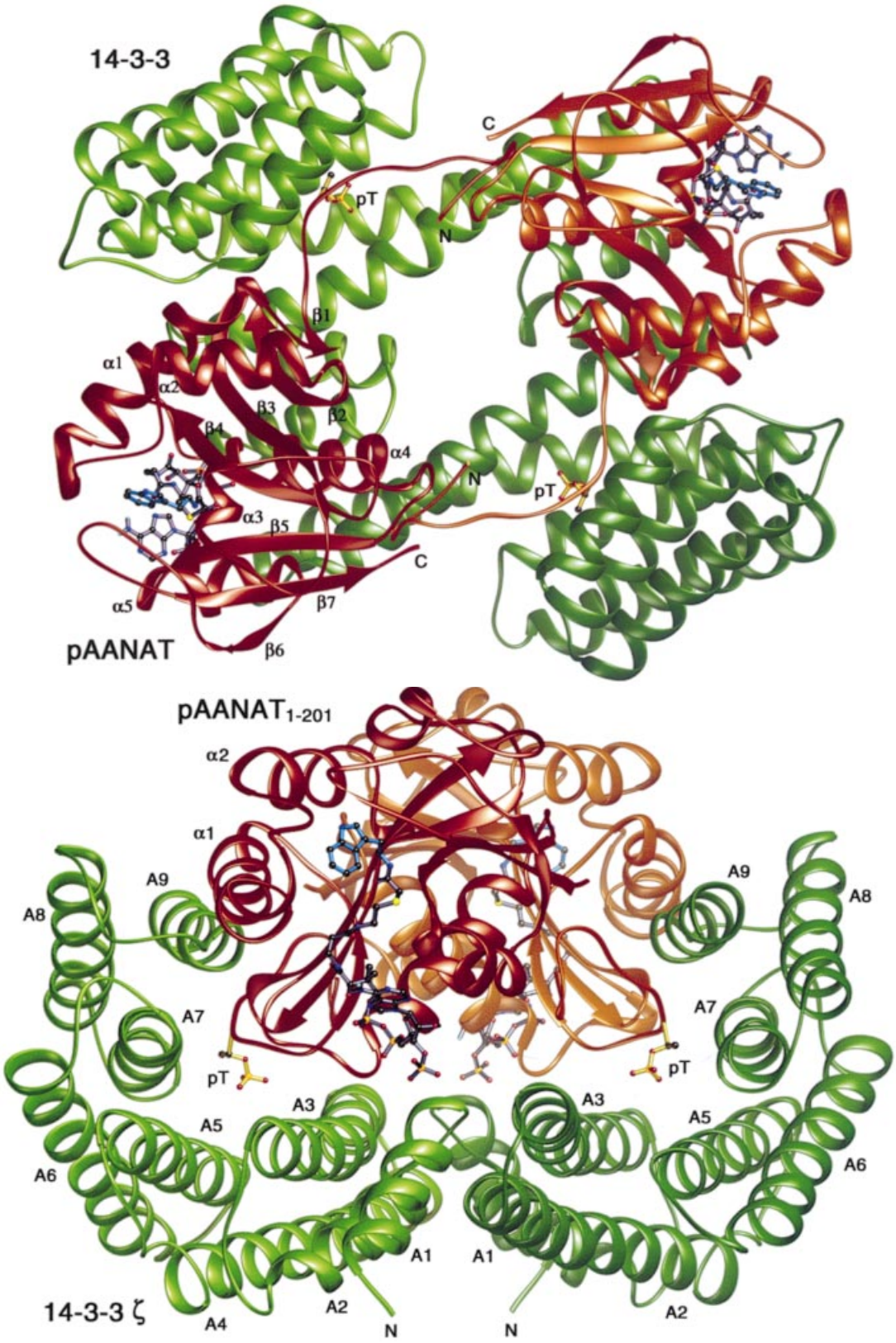
Chromatographic studies of ovine pineal homogenates suggest that the 14-3-3 ε and ζ isoforms may be preferentially involved in the physiological formation of the AANAT–14-3-3 complex, even though these isoforms are not dominant [4,7]. Such partner selection could be determined by 14-3-3 residues involved in binding. However, structural analysis does not support this, because 34 of the 37 14-3-3 ζ residues involved in binding of AANAT are common to all other 14-3-3 isoforms in the databases; the remaining contact residues represent conservative substitutions [8]. Accordingly, the molecular basis for the selection of an AANAT-binding partner remains unclear, although it is possible that these minor differences and others involving interior residues might influence surface-charge topology of the central groove and that this determines partner selection.

As indicated above, chromatographic studies using expressed proteins indicated that each complex contained one molecule of AANAT and one 14-3-3 dimer (1:1 dimer) [7]. However, two molecules of AANAT are bound to a 14-3-3 dimer in the crystal (2:1 dimer) [8]. The reason for the difference is not clear. One contributing factor might be that the crystals were formed using a truncated form of the enzyme, $AANAT_{1–201}$, whereas full-length $AANAT_{1–207}$ was used in the chromatographic studies. The deleted region contains the C-terminal PKA site, which might participate in physiological complex formation through interactions with the unoccupied amphipathic groove of the second 14-3-3 monomer, thereby preventing binding of a second AANAT molecule. This conflict would not occur with truncated AANAT, and would permit binding of a second AANAT resulting in a 2:1 dimer complex. Molecular modelling based on the 2:1 dimer complex indicates that, if present, the phosphorylated C-terminal segment of AANAT, whose N-terminal phosphopeptide is bound by one of the 14-3-3 monomers, could easily reach the phosphopeptide binding groove of the other 14-3-3 monomer. This would prevent the binding of the second AANAT molecule to the 14-3-3 dimer,

Figure 3

Structure of the 14-3-3 ζ–phospho-AANAT$_{1–201}$ complex

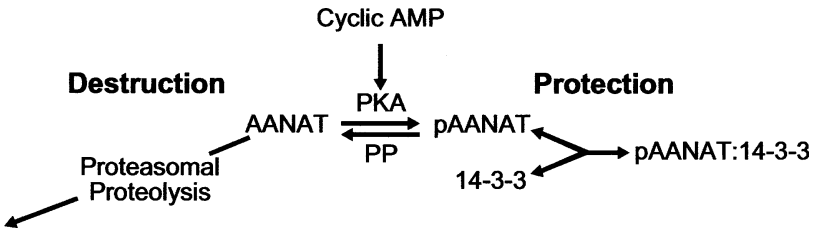
Phospho-AANAT$_{1–201}$ (pAANAT$_{1–201}$) molecules are in brown, phosphorylated Thr-31 (pT) is in yellow, and the bisubstrate analogue is in blue. The 14-3-3 ζ dimer is in green. Top panel: 'top' view looking down into the central groove of 14-3-3 ζ. Bottom panel: 'end' view through the central groove of the 14-3-3 ζ dimer [8].



Scheme 1

Regulation of AANAT by cAMP

The regulation of AANAT protein by cAMP is a balance between 'Destruction' (shown on the left) and 'Protection' (shown on the right). In the absence of cAMP, AANAT does not bind to 14-3-3 and is rapidly destroyed by proteasomal proteolysis. cAMP shifts the balance to the right by directing phosphorylation of AANAT (pAANAT), which triggers binding to 14-3-3. Binding is reversible. When AANAT is bound to 14-3-3, pAANAT is protected against destruction and is activated. The balance shifts towards 'Destruction' when cAMP levels drop, because existing molecules of pAANAT disassociate from the complex and reassociation is blocked by dephosphorylation by protein phosphatase (PP); unbound AANAT is then destroyed.



providing an obvious explanation for the observed stoichiometry in solution.

Effect of complex formation on AANAT biology

Kinetics

As described above, the binding of 14-3-3 restricts the flexibility of loop 1 and stabilizes the binding pockets [7,8]. Stabilization of the arylalkylamine-binding pocket decreases the K_m for arylalkylamines (higher affinity) by 10-fold. This would be especially advantageous in the cell, because it would allow the enzyme to maintain melatonin production when serotonin levels are reduced, as is the case at night when acetylation lowers total serotonin levels as much as 95 %. The reduction in K_m is associated with a decrease in V_{max}, probably reflecting hindrance of reactant flux through the active site. This is of no physiological significance, because it occurs only at concentrations of arylalkylamines that are not physiologically relevant.

Stability

cAMP-dependent phosphorylation of AANAT favours stability [7,10], which appears to reflect binding to 14-3-3 proteins, conferring protection and preventing proteasomal proteolysis. Dephosphorylation of AANAT by phosphatase is reduced in the presence of 14-3-3 and proteolytic cleavage of the N-terminal flanking region is also prevented [7]. The precise fate of unphosphorylated AANAT is not known, other than its destruction by proteasomal proteolysis [11–13,32]. This might involve a conserved N-terminal lysine (Lys-10); such residues are thought to be involved in ubiquitination and subsequent proteasomal proteolysis.

Summary

AANAT is the first enzyme in the serotonin/*N*-acetylserotonin/melatonin pathway and plays a dual role in photoneuroendocrine transduction by the pineal gland as the molecular interface between regulation and melatonin production. This enzyme exhibits large day/night changes in activity and in abundance, and it disappears rapidly at night following exposure to light. These changes are regulated by cAMP, acting through a cAMP-operated 14-3-3-binding switch (outlined in Scheme 1).

Binding of AANAT to 14-3-3 proteins yields a relatively stable complex that enhances the kinetic efficiency of AANAT and shields it against proteolytic destruction. The complex is reversible and free phosphorylated AANAT can either bind to 14-3-3 or be dephosphorylated, in which case it will be subject to rapid proteolytic destruction. Through these actions 14-3-3 proteins can be seen as being of fundamental importance through melatonin-mediated effects on circadian and seasonal physiology [2,3,18,36].

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Plant 14-3-3 protein families: evidence for isoform-specific functions?

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Abstract

14-3-3 proteins regulate a wide range of target proteins via direct protein–protein interactions. The target-binding domain in 14-3-3 proteins is highly conserved, suggesting similar biochemical properties for all 14-3-3s. However, higher eukaryotes possess multiple 14-3-3 genes, and these genes exhibit diverse patterns of gene expression within any one organism. This tends to suggest specific functions for particular genes. Some biochemical data suggest 14-3-3 isoform-specific protein–protein interactions, whereas other studies conclude that apparent isoform-specificity is the result of differences in expression patterns rather than in the biochemical properties of 14-3-3 isoforms. Here we discuss evidence that demonstrates that the expression levels of 14-3-3 proteins in cells are important for regulating the activity of their target proteins, and further that the elimination of individual 14-3-3 isoforms can result in detectable phenotypes. We also examine

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Abbreviation used: BAD, Bcl2-antagonist of cell death protein.
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